4-19-2002

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Recommended Citation

Bullard, J. M., Williams, J. C., Acker, W. K., Jacobi, C., Janjic, N., & McHenry, C. S. (2002). DNA Polymerase III Holoenzyme from Thermus thermophilus Identification, Expression, Purification of Components, and Use to Reconstitute a Processive Replicase. Journal of Biological Chemistry, 277(16), 13401–13408. https://doi.org/10.1074/jbc.M110833200

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 16, Issue of April 19, pp. 13401–13408, 2002
Printed in U.S.A.

DNA Polymerase III Holoenzyme from *Thermus thermophilus* Identification, Expression, Purification of Components, and Use to Reconstitute a Processive Replicase*

Received for publication, November 12, 2001, and in revised form, January 28, 2002
Published, JBC Papers in Press, January 31, 2002, DOI 10.1074/jbc.M110833200

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DNA replication in bacteria is performed by a specialized multicomponent replicase, the DNA polymerase III holoenzyme, that consist of three essential components: a polymerase, the β sliding clamp processivity factor, and the DnaX complex clamp-loader. We report here the assembly of the minimal functional holoenzyme from *Thermus thermophilus* (*Tth*), an extreme thermophile. The minimal holoenzyme consists of α (pol III catalytic subunit), β (sliding clamp processivity factor), and the essential DnaX (τ/γ), δ and δ′ components of the DnaX complex. We show with purified recombinant proteins that these five components are required for rapid and processive DNA synthesis on long single-stranded DNA templates. Subunit interactions known to occur in DNA polymerase III holoenzyme from mesophilic bacteria including δ-δ′ interaction, δδ′-τ/γ complex formation, and α-τ interaction, also occur within the *Tth* enzyme. As in mesophilic holoenzymes, in the presence of a primed DNA template, these subunits assemble into a stable initiation complex in an ATP-dependent manner. However, in contrast to replicative polymerases from mesophilic bacteria, *Tth* holoenzyme is efficient only at temperatures above 50 °C, both with regard to initiation complex formation and processive DNA synthesis. The minimal *Tth* DNA polymerase III holoenzyme displays an elongation rate of 350 bp/s at 72 °C and a processivity of greater than 8.6 kilobases, the length of the template that is fully replicated after a single association event.

DNA replication in all biological systems is performed by specialized multiprotein replicases (1, 2). Cellular replicases consist of three major subassemblies: a sliding clamp processivity factor, a clamp loader, and a specialized polymerase. Replicases, especially bacterial replicases, are rapid and processive consistent with the requirement for them to synthesize a several megabase genome from a single origin in less than one hour.

In the prototypic *Escherichia coli* replication system, a key determinant of processive DNA synthesis is the interaction between the β processivity factor and pol III[1] (3, 4). The dimeric β subunit is a bracelet-shaped molecule that clamps around DNA permitting it to rapidly slide along duplex DNA without dissociating (5). β binds to the pol III α subunit through protein-protein contacts preventing the polymerase from dissociating from the template, ensuring high processivity. Efficient loading of the β subunit onto DNA requires ATP-dependent opening and closing of the clamp by the DnaX complex. The DnaX complex contains the essential DnaX, δ and δ′ subunits plus two ancillary proteins, χ and ψ (6–9). The *dna*X gene encodes two proteins, γ and τ, by programmed ribosomal frameshifting (10–15). Both τ and the shorter γ product share ATP-binding domain I, domain II, and domain III that is responsible for DnaX oligomerization, χ-ψ binding, and binding of δ-δ′ (16–19). τ contains two unique domains. τ domain IV forms a link with the DnaB helicase and domain V binds pol III (17, 20, 21). Pol III consists of α, the catalytic polymerase subunit associated with the ε 3′ → 5′ exonuclease, and θ (22). Pol III gains its special replicative properties by its ability to associate with β and τ through interactions enabled by sequences located in the carboxyl-terminal third of the α subunit (23–26).

The *E. coli* holoenzyme is held together by multiple protein-protein interactions among subunits with the stoichiometry $(\alpha\epsilon\theta)_2\tau_2\gamma\delta\delta'\chi\psi(\beta_2)_2$[2] (27). Pol III (αεθ) forms a stable, isolable complex held together by α-ε and ε-θ interactions (22, 28–30). The α and ε subunits enhance the activity of one another (30–32). Within the DnaX complex, three DnaX subunits form a pentameric core with structurally related proteins, δ and δ′ (19, 27, 33). γ binds χ-ψ and appears to reside adjacent to δ′ within the pentameric ring that makes up the core of the DnaX complex (16, 19, 33, 34). δ′ and δ associate with one another free in solution and within DnaX complex through interaction of their COOH-terminal domain IIIs (19, 33, 35, 36).

DNA polymerase I-like polymerases have been well characterized from thermophilic eubacteria (37, 38). Although these enzymes provide the basis for PCR and related methods (39), they are limited in their processivity and repeatedly dissociate and reassociate with intermediate products during the amplification process. The core of a replicase-like polymerase from a thermophile was isolated by pursuing a minor activity that could be resolved chromatographically from *Tth* DNA polymerase I (40). The purified novel polymerase cross-reacted with a subset of monoclonal antibodies directed against *E. coli* Pol III and was found associated with two proteins that exhibited strong sequence similarity with the γ and τ subunits of *E. coli*

* This work was supported, in part, by Small Business Innovation Research Grant GM54482 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Pol III, DNA polymerase III (αεθ); Pol III holoenzyme, DNA polymerase III holoenzyme; DnaX complex, a complex composed of an oligomer of DnaX (τ and/or γ), δ and δ′; RFII, replicative form II; *Tth*, *Thermus thermophilus*; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

[2] B. Glover, A. Pritchard, and C. McHenry, submitted for publication.

holoenzyme, suggesting the existence of a thermophilic counterpart of the characterized mesophilic pol III holoenzymes.

That *Tth* pol III was found associated with both τ- and γ-sized *dna*X gene products also suggested conservation of the principle of expression of at least two proteins from one *dna*X mRNA (40). A *dna*X-like mRNA was independently isolated by a PCR approach and expressed in *E. coli*, resulting in synthesis of γ- and τ-like proteins (41). It was recently discovered that *Tth* γ and τ are translated from distinct mRNAs that result from transcriptional slippage at a stretch of adenosine residues located at the same site as the *E. coli* translational frameshift site (42).

Encouraged by our detection of the core of the *Tth* replicase we pursued identification of the structural genes that encode the essential subunits of the *Tth* holoenzyme. In this report, we describe the expression and purification of the α, DnaX (τ/γ), δ, δ′, and β subunits of *Tth* holoenzyme and their use to reconstitute a processive thermophilic replicase.

EXPERIMENTAL PROCEDURES

Materials—Antibiotics were purchased from Fisher Scientific. *d*-Biotin was purchased from Sigma. Tryptone and yeast extract were purchased from Difco. Agarose and reagents for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad. Ni-NTA-agarose was purchased from Qiagen. Dialysis membrane (MWCO: 50,000 and 10,000, 22-mm diameter) was purchased from Spectrum Laboratories.

Strains—E. coli strains MGC1030 (*mcr*A, *mcr*B, λ(−), inversion (*rrn*D-*rrn*E), *lex*A3.*omp*T) and AP1.L1 were transformed with plasmids for protein expression. The parent to the AP1.L1 bacterial strain was Novagen BLR bacterial strain [F-, ompT hsdSB(rB- mB-) gal dcm δ(srl-recA)306::Tn10]. AP1.L1 is a phage-resistant version of the BLR strain developed by Dr. Arthur Pritchard (University of Colorado Health Sciences Center, Denver, CO). DH5α (F Φ80*lac*ZΔM15 Δ(*lac*-ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, mK^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1) was transformed with plasmids for amplification of vectors.

Buffers—Buffer E is 50 mM Tris-HCl (pH 7.5), 40 mM KCl, 7 mM $MgCl_2$, 10% glycerol, and 7 mM β-mercaptoethanol. Buffer W is 50 mM Tris-HCl (pH 7.5), 1 M KCl, 7 mM $MgCl_2$, 10% glycerol, 7 mM β-mercaptoethanol, and 10 mM imidazole. TDB buffer is 50 mM HEPES (pH 7.5), 20% glycerol, 0.02% Nonidet P-40, and 0.2 mg/ml bovine serum albumin. HG.04 buffer is 20 mM HEPES (pH 7.5), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 7.0 mM β-mercaptoethanol, and 10% glycerol. ICX buffer is 25 mM HEPES (pH 7.5), 100 mM NaCl, 8 mM MgOAc, and 5% glycerol. TBST buffer contained 200 mM Tris-HCl (pH 7.5), 1.5 M NaCl, 0.2% Tween 20 (v/v).

General Molecular Cloning Procedures—All intermediate plasmids were transformed into DH5α. Amp^R colonies were selected and the plasmids were screened for gain or loss of the appropriate restriction sites. The sequences of inserted DNA were confirmed by DNA sequencing. Ligations were performed in the presence of T4 DNA ligase and ATP. Restriction enzymes were used according to the manufacturer's instructions.

Construction of Starting Vectors—The expression vectors utilized in this study were constructed from two starting vectors: pA1-NB-AvrII and pA1-NB-AgeI. To construct pA1-NB-AvrII, pDRK-N(M), a plasmid designed for expression of proteins with an amino-terminal tag was used as the starting plasmid (24). The amino-terminal tag is composed of a 30-amino acid peptide that is biotinylated *in vivo* and contains a hexahistidine sequence. The construct also contains a pBR322 origin of replication, a gene expressing the $laqI^Q$ repressor protein, and a semisynthetic *E. coli* promoter (pA1) that is repressed by the $lacI^Q$ repressor. Oligonucleotides 5′-CTAGGAAAAAAAAAGGTACCAAAAA-AAAAGGCCGGCCACTAGTG-3′ and 5′-TCGACACTAGTGGCCGGCC-TTTTTTTTTGGTACCTTTTTTTTTC-3′ were annealed to form a duplex with sticky ends (*Avr*II and *Sal*I), and inserted into the *Avr*II/*Sal*I-digested pDRK-N(M). The insertion of these annealed DNA fragments converted the polylinker following the fusion peptide from *Pst*I-*Avr*II-*Dra*III-*Sal*I to *Pst*I-*Avr*II-spacer-*KpnI-spacer-FseI-Spe*I-*Sal*I.

Construction of pA1-NB-AgeI was accomplished by modification of pA1-NB-AvrII whereby the polylinker in pA1-NB-AvrII that contained the restriction sites *Pst*I-*Avr*II-*Kpn*I-*Fse*I-*Spe*I was replaced with a polylinker containing the restriction sites *Pst*I-spacer-*Age*I-*Bam*HI-*Sac*II-spacer-*Nco*I-*Spe*I. First, a *Bam*HI site upstream of the polylinker was destroyed. This was accomplished by digesting pA1-NB-AvrII with *Bam*HI and removing the sticky ends created by filling in with Klenow fragment. The blunted ends of the DNA were resealed. This plasmid was named pA1-NB-AvrII(*Bam*HI$^-$) and was digested with *Pst*I/*Spe*I restriction enzymes. This removed the polylinker containing the restriction sites *Pst*I-*Avr*II-*Kpn*I-*Fse*I-*Spe*I. Oligonucleotides 5′-GAAAA-AAAAAACCGGTGGATCCGCGGAAAAAAAACCATGGA-3′ and 5′-CT-AGTCCATGGTTTTTTTTCCGCGGATCCACCGGTTTTTTTTTTCTG-CA-3′ were annealed and formed a DNA duplex with the sticky ends corresponding to *Pst*I and *Spe*I restriction sites. This annealed DNA duplex was inserted into the *Pst*I/*Spe*I-digested pA1-NB-AvrII thereby forming pA1-NB-AgeI.

Construction of Expression Vectors—Expression of *Tth dna*E (α subunit) was accomplished by the insertion of the *dna*E gene into pA1-NB-AvrII. The *dna*E gene was amplified by PCR of the coding region from a *Bam*HI fragment (4.9 kb) of *Tth* genomic DNA containing the *dna*E gene cloned into a pBluescript IIKS+ phagemid vector. The forward primer 5′-GAATTCCTAGGCCGCAAACTCCGCTTC-3′ adds an *Avr*II site to the 5′ end of the *dna*E gene so that the actual PCR product excludes the ATG start codon and begins at codon 2. The *Avr*II restriction site is adjacent to codon 2 and positions the 5′ portion of the *dna*E gene in the same reading frame with the NH_2-terminal fusion peptide coding sequences. The underlined sequence indicates the region of the primer complementary to the gene of interest here and below. The reverse primer (5′-GTGCTCGCGCAGGATCTCCCGGTCAATC-3′) was designed so that it was downstream of a *Kpn*I restriction site within *Tth dna*E (the *Kpn*I site is ~320 bases downstream of the start codon). The resulting PCR product was digested with *Pst*I and *Kpn*I and ligated into pAI-NB-AvrII that had been digested with the same two restriction enzymes forming pA1-NB-TE(5′). A 3454-bp *Kpn*I/*Fse*I fragment representing the 3′-end of the *Tth dna*E gene was isolated from the 4.9-kb *Bam*HI *Tth* genomic DNA fragment cloned into the pBluescript IIKS+ plasmid and inserted into the corresponding sites of pA1-NB-TE(5′). This plasmid (pA1-NB-TE) contained the entire *Tth dna*E gene fused to a sequence encoding the amino-terminal tag.

*Tth dna*X was inserted into an expression vector by the same procedure as used for *dna*E. A PCR reaction was designed to amplify a fragment containing the 5′ end of *dna*X from a plasmid containing a 7.1-kb cloned fragment of *Tth* genomic DNA which contained the *Tth dna*X gene. The forward primer (5′-AACTGCAGAGCGCCCTCTACCG-3′) added a *Pst*I site to the 5′ end of the *dna*X gene so that the actual PCR product excludes the ATG start codon and begins at codon 2. The *Pst*I restriction site positions the 5′ portion of the *dna*X gene in the same reading frame with the N-terminal fusion peptide coding sequences. The reverse primer (5′-CGGTGGTGGCGAAGACGAAGAG-3′) was designed so that it is downstream of the *Bam*HI restriction site within *Tth dna*X (the *Bam*HI site is ~318 bases downstream of the start codon). This PCR product was digested with *Pst*I and *Bam*HI and ligated into pAI-NB-AgeI which had been digested with the same two restriction enzymes forming pA1-NB-TX(5′). The 3′ region of the *dna*X gene was removed from the 7.1-kb fragment by digestion with *Bam*HI and *Spe*I and inserted into the precursor plasmid pA1-NB TX5′ that had been digested with the same two restriction enzymes. This formed the plasmid pA1-NB-TX, which contained the full-length *Tth dna*X gene fused to the sequence encoding the amino-terminal tag.

The *Tth hol*A gene that encodes the δ subunit was inserted into pA1-NB-AvrII to be expressed fused to the amino-terminal tag. The *hol*A gene was amplified by PCR using *Tth* genomic DNA as a template. The forward/sense primer (5′-GAATTCTGCAGGTCATCGCCTTCACC-G-3′) added a *Pst*I site to the 5′ end of the gene so that the actual PCR product excludes the ATG start codon and begins at codon 2. The *Pst*I site adjacent to codon 2 places the *hol*A gene in the same reading frame with the NH_2-terminal fusion peptide. The reverse primer (5′-AGATC-TGGTACCTCATCAACGGGCGAGGCGGAG-3′) added an additional TGA (stop codon) to the end of the gene giving two stop codons in tandem and a *Kpn*I restriction site was added in the non-complementary region of the primer for insertion into the vector. The PCR product was digested with *Pst*I and *Kpn*I restriction enzymes and inserted into pA1-NB-AvrII digested with the same enzymes. This formed the plasmid pA1-NB-TD, which contained the full-length *Tth hol*A gene fused to the DNA sequence encoding the amino-terminal tag.

The *Tth hol*B gene which encodes δ′ subunit was cloned into pA1-NB-AgeI to be expressed fused to the amino-terminal tag. The *hol*B gene was amplified by PCR using *Tth* genomic DNA as a template. The forward/sense primer (5′-GAATTCTGCAGGCTCTACACCCGGCT-CACCC-3′) added a *Pst*I site to the 5′ end of the gene so that the actual PCR product excludes the ATG start codon and began at codon 2. The *Pst*I site adjacent to codon 2 places the *hol*A gene in the same reading

frame with the NH_2-terminal fusion peptide. The reverse primer (5′-GGACACTAGTTCATCATGTCTCTAAGTCTAA-3′) was complementary to the 3′ end of the *hol*B gene and added an additional TGA (stop codon). In addition, a *Spe*I restriction site was added in the non-complementary region of the primer for insertion into the vector. The PCR product containing the entire *Tth hol*B gene was digested with *Pst*I/*Spe*I restriction enzymes and inserted into pA1-NB-AgeI digested with the same enzymes. This formed the plasmid pA1-NB-TD′, which contained the full-length *Tth hol*B gene fused to the DNA sequence encoding the amino-terminal tag.

To express *Tth* β subunit, a PCR fragment containing the *dna*N gene was amplified from genomic DNA. The forward primer (5′-AACTGCAGAACATAACGGTTCCCAAGAAACTCC-3′) added a *Pst*I restriction site to the 5′ end adjacent to codon 2, so that when this fragment was inserted into pA1-NB-Age1, the *dna*N gene was in the same reading frame with the sequence encoding the amino-terminal tag. The reverse primer 5′-GAGCAGCTAGCCTACTAGACCCTGAGGGGCACCAC-3′) was designed so that an additional stop codon was added in the non-complementary region producing two stop codons in tandem. The non-complementary region of the reverse primer also contains an *Nhe*I restriction site. The PCR reaction resulted in a product that contained the entire *Tth dna*N gene. The PCR product was digested with *Pst*I and *Nhe*I and inserted into *Pst*I/*Nhe*I-digested pA1-NB-AgeI forming pA1-NB-TN. The correct DNA sequence of all *Tth* genes cloned into expression vectors was confirmed by DNA sequencing.

Cell Growth and Preparation of Fraction I—Cells were grown, induced, and harvested using a 250-liter fermentor as described (43), with the exception that ampicillin (100 μg/ml) and *d*-biotin (10 μM) was added at induction. Lysis of 400 g of cells and preparation of Fraction I for each of the subunits was performed as described by Cull and McHenry (44). The Fraction I supernatants contained the following levels of protein: α (25 g), β (22 g), DnaX (25 g), δ′ (20 g), and δ (39 g).

Purification of Proteins—As an initial purification step, many endogenous *E. coli* proteins can be removed by adding ammonium sulfate to concentrations that cause the protein of interest (and some endogenous proteins) to precipitate, while other proteins remain in solution. Therefore, the concentration of ammonium sulfate (expressed as percent saturation at 4 °C) in which >80% of *Tth* target proteins and minimal amounts of *E. coli* contaminating proteins precipitate was determined for each subunit by SDS-polyacrylamide electrophoresis. The *Tth* subunits were precipitated using ammonium sulfate concentration of 45% saturation for α, 40% for τ/γ, δ, and β, and 35% for δ′ and were collected by centrifugation (23,000 × *g*, 45 min, 0 °C).

Ammonium sulfate pellets were dissolved in 125 ml of buffer E using a Dounce homogenizer. Samples were clarified by centrifugation (16,000 × *g*) resulting in Fraction II. Fraction II contained the following levels of protein: α (3000 mg), β (2600 mg), DnaX (1100 mg), δ′ (620 mg), and δ (4400 mg). Fraction II was added to 60 ml of a 50% slurry of Ni-NTA resin in buffer E and agitated for 1.5 h at 4 °C and then poured into a column (2.5 × 5 cm). The column was washed with 300 ml of buffer W at a flow rate of 0.5 ml/min. Tagged *Tth* proteins were eluted in 300 ml (10 column volumes) of buffer E containing a 10–200 mM imidazole-HCl (pH 7.5) gradient. The eluate was collected in 150 × 2-ml fractions and the protein concentration for each fraction was determined. Fractions making up the upper one-half of the protein concentration peak were pooled. The proteins were >90% pure based on densitometric analysis of Coomassie Blue-stained SDS-polyacrylamide gels. Following purification, the proteins were dialyzed into HG.04 buffer (Spectra/Por dialysis tubing, 11.5-mm diameter, 3000 MWCO), rapidly frozen in liquid nitrogen, and stored at −80 °C. The resulting Fraction IIIs contained the following levels of activity and protein: α (50 mg, 9.0×10^7 units, 1.8×10^6 units/mg), β (325 mg, 5.5×10^8 units, 1.7×10^6 units/mg), DnaX (85 mg, 2.4×10^8 units, 2.8×10^6 units/mg), δ′ (35 mg, 6.0×10^8 units, 17.0×10^6 units/mg), and δ (325 mg, 45.0×10^8 units, 14.0×10^6 units/mg). One unit of activity is 1 pmol of total deoxyribonucleotide incorporated per min at 60 °C in the M13Gori reconstitution assay.

Protein Assays, SDS-PAGE, Biotin, and Western Blots—Protein concentrations were determined by the method of Bradford (45) using bovine serum albumin as a standard. For SDS-PAGE, proteins were resolved by electrophoresis on 10% SDS-polyacrylamide gels (14 × 16 × 0.75 cm) for 2.5 h at 250 V, and visualized by staining with Coomassie Brilliant Blue. Proteins containing *d*-biotin were detected by biotin blots using phosphatase-conjugated streptavidin. Proteins resolved on an SDS-polyacrylamide gel were electroblotted onto a nitrocellulose membrane (Immobilon-P, Millipore) using a Hoefer™ SemiPhor transfer apparatus at constant voltage (30 V) in 12 mM Tris base (pH 8.5), 96 mM glycine, 0.01% SDS (w/v), and 20% methanol (v/v) for 60 min at room temperature. Membranes were blocked in TBST buffer containing 5% non-fat dry milk (w/v) for 1 h at room temperature. Membranes were then rinsed with TBST and incubated in 2 μg/ml alkaline phosphatase-conjugated streptavidin (Pierce Chemical Co.) in TBST for 1 h at room temperature. Following extensive washing with TBST, membranes were developed using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium One Component System (Pierce Chemical Co.).

Western blot analysis of DnaX proteins was performed using the same method as biotin blots except that monoclonal antibodies elicited to NH_2-terminal-tagged DnaX (Tissue Culture/Monoclonal Antibody Facility, University of Colorado Health Sciences Center) were used instead of streptavidin. The antibodies selected for Western blotting experiments were specific for DnaX protein sequence and did not recognize hexahistidine and biotinylation sequences on other NH_2-terminal-tagged *Tth* subunits. Membranes were developed using goat anti-mouse antibodies conjugated to alkaline phosphatase (Bio-Rad).

Reconstitution Assays—We employed a modified version of the M13Gori assay in which DNA synthesis on a long single-stranded circular template (M13Gori) primed by an RNA primer is used to measure the activity of DNA holoenzyme (46). G4 origin-specific primers were formed by the *E. coli* DnaG primase on *E. coli* single-stranded DNA-binding protein-coated M13Gori DNA. Each 19-μl assay contained 0.06 pmol of M13Gori DNA (about 500 pmol total nucleotide), 50 ng of DnaG primase, 1.6 μg of single-stranded DNA-binding protein, 10 mM magnesium acetate, 200 μM ATP, GTP, CTP, and UTP, 48 μM dATP, dGTP, and dCTP, and 18 μM [^{3}H]dTTP (100 cpm/pmol). In some cases, M13Gori single-stranded DNA was primed by a 30-mer DNA oligonucleotide (5′-AGATTACTCTTGATGAAGGTCAGCCAGCCT-3′) in reactions conducted in the absence of single-stranded DNA-binding protein or DnaG. The DNA-primed M13Gori was prepared by incubating 0.78 μM 30-mer DNA with 0.52 μM M13Gori in 10 mM HEPES buffer (pH 7.5) containing 100 mM NaCl, heating to boiling, and cooling slowly to room temperature. The RNA or DNA primed template mixtures were used in all M13Gori assays and are referred to as the primed template mixture. The *Tth* subunits α, τ/γ, δ, δ′, and β were diluted to the desired concentration in TDB buffer in a total volume of 6 μl and combined with 19 μl of the primed template mixture to yield a 25-μl reaction. Following mixing, the reaction contents were incubated for 5 min at 60 °C. The reaction was terminated by placing the reaction tube on ice and adding 2 drops of 0.2 M sodium pyrophosphate and 0.5 ml of 10% trichloroacetic acid. The solution was filtered under vacuum through Whatman GF/C glass microfiber filters. The filters were then washed with 3 assay tube volumes (3 × 5 ml) of 1 M HCl, 0.2 M sodium pyrophosphate, and 1 assay tube volume (5 ml) of 95% EtOH and dried using a heat lamp. The amount of radiolabeled nucleotides incorporated was quantified by scintillation counting. One unit of activity is 1 pmol of total deoxyribonucleotide incorporated per min at 60 °C.

Protein-Protein Interactions Determined by Gel Filtration—Gel filtration analysis of the interaction of DNA pol III holoenzyme subunits was preformed using a Sephacryl™ S-200 (Amersham Bioscience) column (0.7 × 30 cm) equilibrated with HG.04 buffer. All proteins and protein mixtures were incubated for 5 min at 60 °C in 300 μl of HG.04 buffer prior to loading onto the column. To ensure that the runs were uniform, the sample was gently pipetted onto the resin, pumped into the resin, then 1 ml of buffer was gently added onto the top of the resin and pumped into the resin. The running buffer was then added to the top of the resin and elution was continued. The first three fractions (1 ml each) contained the void volume and all subsequent fractions contained 300 μl. Fractions were analyzed in 10% SDS-polyacrylamide gels stained with Coomassie Brilliant Blue and scanned using a Molecular Dynamics laser densitometer. The stoichiometry of a candidate complex formed on mixing α, τ/γ, and δδ′ subunits was determined from Coomassie Blue stain intensities corrected for the differences in molecular masses of the subunits (but not differences in subunit-specific dye binding) using 141 kDa for α, 61.9 for τ, 53.6 kDa for γ, 36.2 kDa for δ, and 33.0 kDa for δ′.

Gel filtration analysis of δ and δ′ alone was performed with 200 and 100 μg of protein, respectively (in 300 μl of HG.04 buffer). For δ and δ′ interaction analysis, a mixture containing 150 μg of each protein was used. Gel Filtration of τ/γ alone was performed using 170 μg of protein. Analysis of interactions of τ/γ with δ and δ′ was performed using 85 μg of δ, 70 μg of δ′, and 170 μg of τ/γ. α (75 μg) was gel filtered alone. The interaction between α and τ/γ was analyzed using 55 μg of α and 170 μg of τ/γ. The α(τ/γ)δδ′ complex formation was analyzed using 40 μg of α, 100 μg of τ/γ, and 55 μg of both δ and δ′. DNA synthesis activity of eluted fractions was determined using holoenzyme reconstitution assays where all of the subunits were present in the reconstitution assay mixture except those that were loaded onto the gel filtration column. 4

μl of a mixture of all subunits except the protein(s) being analyzed were added to 19 μl of the primer/template mixture (see "Reconstitution Assays"). To this mixture, 2 μl taken from the gel filtration fractions were added and the sample was assayed as described above.

Gel filtration of protein standards (Amersham Bioscience) was carried out to determine elution positions of various molecular weight proteins. Each protein standard contained 500 μg of each protein in 300 μl of HG.04 buffer. Standards were chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), and ferritin (440 kDa). Positions of the protein standards in the elution profiles were determined by protein assays.

Initiation Complex Formation—Initiation complexes were formed by mixing 150 pmol of α, 54 pmol of $DnaX_4$ (tetramer is the presumed molecular species (41)), 264 pmol of δ, 264 pmol of δ', 167 pmol of β_2 (presumed dimer (47)), and 4 pmol of DNA-primed M13Gori single-stranded DNA in 240 μl of ICX buffer containing 10 mM dithiothreitol and 1 mM ATP (or dATP or ATPγS). The mixture was incubated for 5 min at 30 or 60 °C and 200 μl was applied to an HR 10/30 Superose 6 column (Amersham Bioscience) equilibrated with ICX buffer. Fractions (0.5 ml) were collected, maintained on ice, and analyzed by SDS-PAGE. Activity assays for the eluted initiation complex were carried out by adding 3 μl of a mixture containing dATP, dGTP, dCTP, and [^{3}H]dTTP (100 cpm/pmol) to 22 μl from each fraction (final concentration of all four deoxynucleotide triphosphates was 48 μM), incubating at 60 °C for 5 min and determining the amount of acid precipitable radioactivity.

Elongation Rate and Processivity Determinations—The elongation rate of the holoenzyme was measured at various temperatures by determining the time required to fully extend the primer over the entire length of the M13Gori template (8623 nucleotides) starting with pre-formed initiation complexes. M13Gori pre-primed with DnaG primase in the absence of deoxyribonucleotides was used as a template. Initiation complexes were formed by mixing 16 μl of the RNA-primed M13Gori (500 pmol, total nucleotide) with 5 μl of *Tth* α, $DnaX_4$, δ, δ', and β_2 subunits (2, 1, 2, 2, and 2 pmol, respectively) in TDB buffer and incubating the mixture at 60 °C for 1 min. DNA synthesis was initiated by adding initiation complex (21 μl) to 4 μl of 0.3 mM dGTP, dCTP, dTTP, and [$\alpha-^{32}$P]dATP (12 mCi/mmol) in TDB. To minimize the time required for thermal equilibration, both solutions were incubated at the desired reaction temperature for 1 min prior to mixing. The reaction was quenched by adding 5 μl of ice-cold 250 mM EDTA. Total DNA synthesis was determined by assaying 1 μl of the reaction mixture for acid precipitable radioactivity. DNA products were analyzed by 0.8% alkaline-agarose gel electrophoresis as described (48).

To determine the processivity of *Tth* holoenzyme, a mixture (22 μl) containing M13Gori (500 pmol, total nucleotide) and *Tth* α, $DnaX_4$, δ, δ', and β_2 subunits (2, 1, 2, 2, and 2 pmol, respectively) in TDB buffer were incubated at 60 °C for 2 min to allow formation of initiation complex. A mixture (5 μl) containing 1.2 mM dGTP, dCTP, dTTP, and [$\alpha-^{32}$P]dATP (12 mCi/mmol) was combined with 1 μl of 5 mg/ml solution of activated calf thymus DNA (31) in TDB buffer and incubated at 60 °C for 1 min. The reaction was initiated by combining the two solutions followed by incubation at 60 °C for an additional 45 s. The reaction was quenched by adding 5 μl of ice-cold 250 mM EDTA. DNA products were analyzed by 0.8% alkaline-agarose gel electrophoresis as described (48). The positive control contained no activated calf thymus DNA and the negative control contained no M13Gori DNA. In a control to show that activated calf thymus DNA acts as an efficient competitor in initiation complex formation, the activated calf thymus DNA and M13Gori were incubated at the same time with *Tth* holoenzyme subunits.

RESULTS

Identification, Expression, and Purification of Tth α, τ/γ, δ, δ', and β Subunits—Genes encoding the α, τ/γ, δ, δ', and β subunits of the *Tth* holoenzyme[3] were either identified or confirmed by searching the Tth genome data base[4] for homology with the corresponding genes from *E. coli* (*dna*E, *dna*X, *hol*A, *hol*B, *and dna*N). The identification of the gene encoding α from *Tth* was aided by partial NH_2-terminal and internal peptide fragment sequencing of the pol III α subunit isolated previously from *Tth* extracts (40). The gene encoding τ/γ (*dna*X) was previously identified by a similar reverse genetics approach (40, 41). Sequence comparison of genes for *dna*E, *dna*X, holA, *hol*B, and *dna*N between *Thermus thermophilus* and *E. coli* revealed 40, 31, 27, 27, and 26% identity, respectively. Each of the genes was amplified by PCR from *Tth* DNA, cloned into vectors containing an amino-terminal hexahistidine and biotinylation sequence, sequenced to confirm identity, overexpressed in *E. coli*, and purified to >90% purity as described under "Experimental Procedures." SDS-polyacrylamide gel analysis indicated that the purified subunits migrated with apparent molecular masses of ~135 kDa for α, 66 and 58 kDa for τ and γ, respectively, 42 kDa for β, and 36 kDa for δ and δ' (Fig. 1). The minor band between τ and γ is probably a degradation product of τ, because it is stained along with the major bands with a monoclonal antibody specific for *Tth* DnaX proteins (data not shown). These molecular mass values are in agreement with the calculated values for the NH_2-terminal-tagged subunits of 141 kDa for α, 61.9 for τ, 53.6 kDa for γ, 44.3 kDa for β, 36.2 kDa for δ, and 33.0 kDa for δ'.

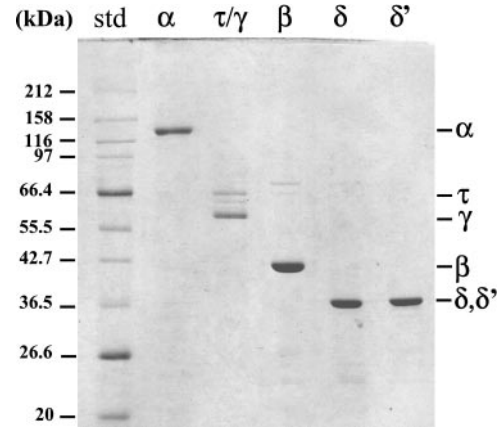

FIG. 1. **Holoenzyme subunits required for reconstitution of a minimal *Tth* replicative polymerase.** Coomassie Blue-stained 10% SDS-polyacrylamide gel of *Tth* α (1.8 μg), τ/γ (3.2 μg), β (3.7 μg), δ (3.7 μg), and δ' (3.4 μg). Molecular mass standards are shown in the lane marked *std*.

Requirement of α, τ/γ, δ, δ', and β Subunits for Processive DNA Synthesis—Our primary goal has been to assemble the minimal thermophilic replicase capable of rapid and processive synthesis of long stretches of DNA. Based on the *E. coli* precedent, we expected that *Tth* α, DnaX (τ/γ), δ, δ', and β would be required. With the availability of these proteins in NH_2-terminal-tagged forms, we used them in initial attempts at reconstitution. We employed a modified form of the standard assay used for the *E. coli* holoenzyme, DNA synthesis on a long single-stranded circular template primed by an RNA primer (49). To achieve efficient DNA synthesis, comparable with that observed with the *E. coli* holoenzyme, *Tth* holoenzyme α, DnaX, δ, δ', and β subunits were required (Table I). In addition, reactions needed to be conducted at 50 °C or higher in contrast

[3] We refer to a *Tth* holoenzyme loosely using the functional definition of the minimal assembly required for processive replication. By analogy to the well studied DNA polymerase III holoenzyme from *E. coli*, we expect that additional biochemically non-essential subunits will be present in the complete *Tth* holoenzyme that may enhance its properties.

[4] Prior to searching the *Tth* database (www.g2l.bio.uni-goettingen.de), *Tth dna*X, *dna*E, and *dna*N had been identified by a variety of "reverse genetic" approaches. For *dna*E and *dna*X, peptide sequences, obtained from proteolytic fragments of the isolated core of *Tth* holoenzyme, were used to direct synthesis of degenerate PCR primers. These primers were used to obtain segments of the *dna*E and *dna*X genes and were confirmed by DNA sequencing. These fragments were used as probes of *Tth* genomic libraries to obtain the full-length genes. For *dna*N, we relied on *dna*A being upstream, an arrangement conserved among most bacteria. A segment of the highly conserved *Tth dna*A gene was obtained by PCR and used as a probe to clone large *Tth* fragments that included *Tth dna*A and *dna*N. We then sequenced downstream of *dna*A to identify the flanking *Tth dna*N gene.

TABLE I
Requirement for Tth holoenzyme subunits in the M13Gori reconstitution assay

Subunits present[a]					DNA synthesis (pmol)[b]
	DnaX	δ	δ′	β	1.0 ± 0.4
α	—[c]	δ	δ′	β	2.3 ± 0.08
α	DnaX	—	δ′	β	1.7 ± 0.04
α	DnaX	δ	—	β	1.5 ± 0.3
α	DnaX	δ	δ′	—	5.6 ± 5.7
α	DnaX	δ	δ′	β	296 ± 17

[a] Subunit concentrations in this experiment contained 0.64, 0.5, 4.0, 2.4, and 2.6 pmol for α, $DnaX_4$, δ, δ′, and $β_2$ subunits, respectively. Average molecular mass of 57.8 kDa was used for DnaX monomer.

[b] The *Tth* subunits α, DnaX (τ/γ), δ, δ′, and β in TDB buffer (6 μl total) were combined in various combinations with 19 μl of RNA-primed template mix containing 0.06 pmol (500 pmol of nucleotides) of M13Gori DNA and dNTPs. The amount of acid-precipitable radioactivity was determined following incubation of the reaction mixture for 5 min at 60 °C.

[c] Absence of subunit.

to 30 °C used for *E. coli* holoenzyme. The amount of each subunit necessary to achieve maximal DNA synthesis under the assay conditions was determined by titrating each subunit into a reaction mixture containing an excess of the other four subunits (Fig. 2). Using these titration profiles as a guide, in subsequent experiments we have used α concentrations of ≥0.024 μM (0.6 pmol in 25 μl), $DnaX_4$ concentrations of ≥0.02 μM (0.5 pmol in 25 μl, using the anticipated tetrameric form of DnaX (41), and average molecular mass of 57.8 kDa for DnaX monomer), δ and δ′ concentrations of ≥0.08 μM (2 pmol in 25 μl) and $β_2$ concentrations of ≥0.1 μM (2.5 pmol in 25 μl, using the anticipated dimeric form of β (47)).

Interaction of Tth Holoenzyme Subunits—We examined protein-protein interactions among holoenzyme subunits by gel filtration. In view of homology between holoenzyme subunits of *E. coli* and *Tth*, we investigated whether some of the known interactions between *E. coli* subunits also occur in *Tth*.

The δ and δ′ subunits of the *Tth* DnaX complex interact with each other as they do in *E. coli* (50). δ′ eluted with a peak at fraction 22 (Fig. 3*A*). δ eluted with a peak at fraction 21[5] (Fig. 3*B*). When δ and δ′ were incubated together at approximately equal molar concentrations, the protein peak was shifted to a δ-δ′ complex with a peak in fraction 19 (Fig. 3*C*). δ and δ′ are similar in size and we were unable to resolve them on our polyacrylamide gels, including gradient gels (data not shown). Therefore, we also tested the DNA synthesis activities of eluting fractions in reconstitution assays where all of the subunits were present in the M13Gori reaction mixture except δ-δ′ (data not shown). These activity data corroborate the protein elution profiles and further support an interaction between the two subunits.

We next asked whether the *Tth dna*X gene products, τ and γ, interact with δ-δ′. Alone, τ and γ eluted together with a peak in fraction 17 (Fig. 3*D*). When τ/γ were incubated with δ and δ′, the elution profile was shifted to a complex with a peak in fraction 16 (Fig. 3*E*), indicating a modest increase in the size of the DnaX proteins and a significant shift of δ-δ′ over the size of the complex alone. This indicates δ-δ′ binds to a DnaX oligomer, just like in *E. coli* (50). In similar experiments not shown here, δ′ alone interacted with τ/γ whereas an interaction of τ/γ with δ was not detectable.

Just as in *E. coli* (23, 51), *Tth* α and τ/γ interact. *Tth* τ/γ and α when filtered alone, both eluted with a protein peak in fraction 17 (Figs. 3*D* and 4*A*). The α-τ/γ complex eluted with a peak at fraction 14 (Fig. 4*B*), indicating a significant size increase upon binding of α to τ. This interaction was observed previously, providing the basis for the original isolation of the DnaX proteins from *Tth* (40). When α, τ/γ, δ, and δ′ were incubated together, all subunits co-eluted in a broad peak encompassing fractions 14–20 (Fig. 4*C*). Since the α-τ/γ complex (Fig. 4*B*) and the τ/γ-δδ′ complex (Fig. 3*E*) separately eluted with a similar profile, it is not possible from the elution profile alone to conclude that a stable complex involving α, τ/γ, and δδ′ is formed. However, quantitative analysis of the Coomassie Blue-stained gel showed that the composition of the complex in the lead fraction 14 (Fig. 4*C*), expressed as the Coomassie Blue stain intensity, normalized to that of α and corrected for molecular masses, but not subunit-specific variation in dye binding, is $α_{1.0}τ_{1.1}\ γ_{1.8}(δδ')_{2.1(total)}$. δ and δ′ subunits could not be quantified separately because of their identical electrophoretic mobilities and were considered as a single species. We also note an enrichment of τ over γ within bound DnaX protein in fraction 14, suggesting it is τ and not γ that binds α, just like in *E. coli*. This observation is consistent with the formation of a

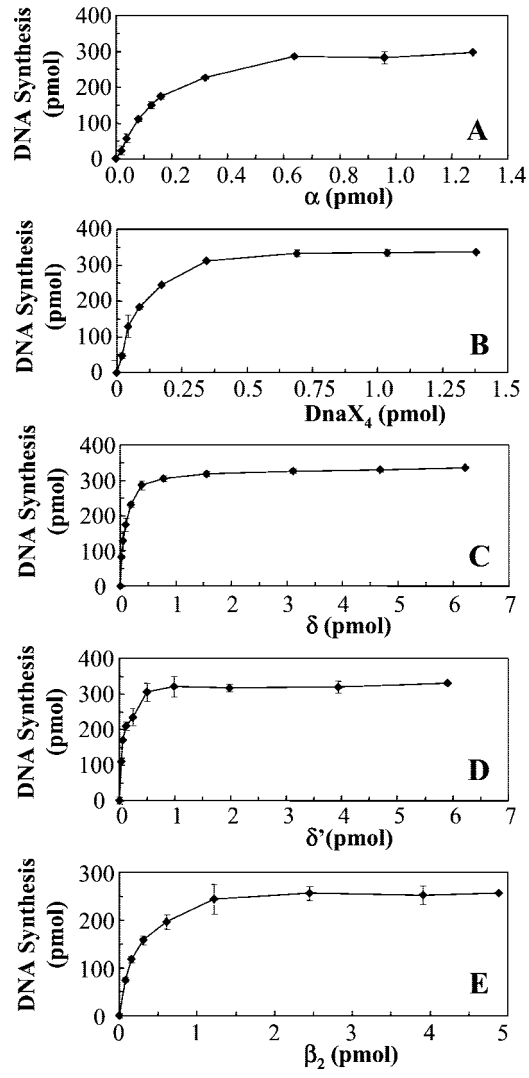

FIG. 2. **Titration of *Tth* holoenzyme subunits into the M13Gori reconstitution assay.** Except when varied as designated in the *X* axis, α, $DnaX_4$, δ, δ′, and $β_2$ subunits were held at saturating concentrations containing 0.64, 0.5, 4.0, 2.4, and 2.6 pmol, respectively. DNA synthesis in the absence of the varied subunit (reported in Table I) was subtracted from all plotted values. *A*, titration of α subunit in the presence of $DnaX_4$, δ, δ′, and $β_2$. *B*, titration of $DnaX_4$ in the presence of α, δ, δ′ and $β_2$. *C*, titration of δ in the presence of α, $DnaX_4$, δ′ and $β_2$. *D*, titration of δ′ in the presence of α, $DnaX_4$, δ, and $β_2$. *E*, titration of $β_2$ in the presence of α, $DnaX_4$, δ, and δ′.

[5] Even though all fractions were not run on the SDS gels used to access gel filtration profiles, we estimated peak tubes by interpolation.

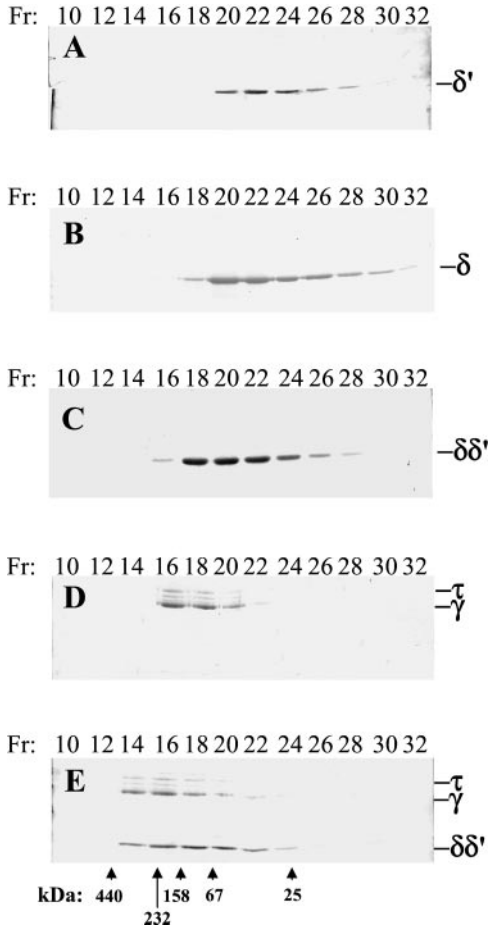

FIG. 3. **Protein interactions between subunits of the *Tth* DnaX complex.** Subunits were mixed to a final volume of 300 μl in HG.04 buffer, incubated at 60 °C for 5 min, and then analyzed by gel filtration as described under "Experimental Procedures." Proteins were visualized by Coomassie Blue staining of 10% SDS-polyacrylamide gels. *A*, δ′ (3.0 nmol). *B*, δ (5.5 nmol). *C*, δ′ + δ (4.5 nmol and 4.1 nmol, respectively). *D*, τ/γ (3.0 nmol, using the average molecular mass of 57.8 kDa). *E*, δ′ + δ + τ/γ (2.1, 2.3, and 3.0 nmol, respectively). Fraction numbers are indicated at the *top* of the gels, the subunits are identified on the *right side* of the gel, and the elution positions of molecular mass markers are shown at the *bottom*. In *panels C* and *E*, δ′ and δ co-migrate on SDS-polyacrylamide gels.

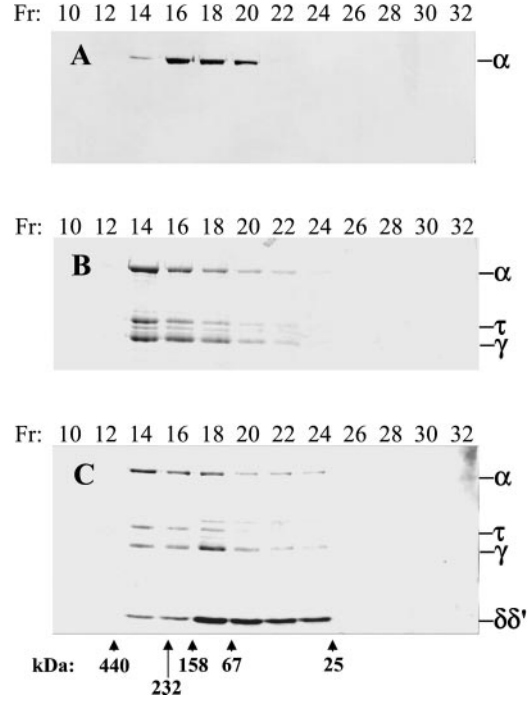

FIG. 4. **Protein interactions between α and DnaX proteins and between α and the DnaX complex.** Subunits were mixed to a final volume of 300 μl in HG.04 buffer, incubated at 60 °C for 5 min and then analyzed by gel filtration as described under "Experimental Procedures." Proteins were visualized by Coomassie Blue staining of 10% SDS-polyacrylamide gels. *A*, α (0.5 nmol). *B*, α + τ/γ (0.4 and 3.0 nmol, respectively). *C*, α + τ/γ + δ′ + δ (0.3 nmol, 2.0, 1.5, and 1.5 nmol, respectively). Fraction numbers are indicated at the *top* of the gel, the subunits are identified on the *right side* of the gel, and the elution positions of molecular mass markers are shown at the *bottom*.

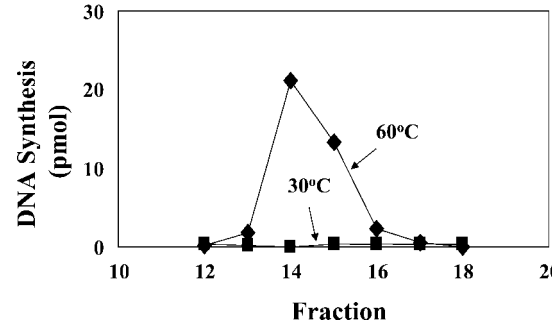

FIG. 5. **Formation of isolable *Tth* holoenzyme initiation complex.** Components of initiation complexes (*Tth* holoenzyme subunits and DNA-primed M13Gori) were mixed and incubated at 30 or 60 °C for 5 min and subjected to gel filtration as described under "Experimental Procedures." *A*, fractions from the HR 10/30 Superose 6 column were assayed for their ability to synthesize DNA following the addition of all four deoxyribonucleoside triphosphates (22 μl of each fraction was assayed). Activity indicates the picomole of nucleotides incorporated during extension of the primer.

complex consisting of $\alpha{\cdot}DnaX_3{\cdot}\delta\delta'$, although further work will be needed to definitively characterize the composition of this and other complexes within the holoenzyme.

Initiation Complex Formation—In *E. coli*, an initiation complex stable to gel filtration is formed upon mixing the holoenzyme with a primed template in an ATP-requiring reaction (52–54). To test whether a similar initiation complex can be formed with *Tth* DNA polymerase III subunits, we incubated α, DnaX, β, δ, and δ′ subunits with M13Gori annealed to a 30-mer DNA primer in the presence of ATP. The calculated T_m of the 30-mer DNA primer at the salt and oligonucleotide concentrations of our assay is ~65 °C (55, 56). Based on activity measurements of each of the eluting fractions, the initiation complex eluted in fractions 14–15 (Fig. 5). In contrast, incubation of subunits with primed template at 30 °C resulted in no initiation complex formation (Fig. 5). The presence of *Tth* α, DnaX, δ, δ′, and β subunits in fractions 14–16 following incubation at 60 °C was confirmed by SDS-polyacrylamide gel electrophoresis of these fractions (data not shown). Following gel filtration, *Tth* replication-competent initiation complex placed on ice loses only about 10% of its DNA synthesis activity over a period of 1 h, providing ample time to complete activity assays. As in *E. coli* (52, 53, 57), initiation complex formation in *Tth* requires ATP, dATP, or ATPγS (Table II).

Elongation Rate—To determine the rate at which the minimal *Tth* holoenzyme synthesizes DNA at different temperatures, we initiated DNA synthesis on pre-formed initiation complex by addition of all four deoxyribonucleoside triphosphates. The reactions were quenched at various times by addition of EDTA. Analysis of the reaction products on alkaline-agarose gels permitted determination of the time required to fully extend the primer over the entire length of the M13Gori template (Fig. 6). At 45 °C, no full-length product (RFII) is formed in 1 min, although 3–5-kb fragments were visible at

TABLE II
Dependence of Tth DNA pol III initiation complex formation on ATP

Nucleotide[a]	DNA synthesis[b]
	pmol
None	2
ATP	37
dATP	39
ATPγS	27

[a] Initiation complexes were formed and subjected to gel filtration as described in the legend of Fig. 5 and under "Experimental Procedures" in the presence or absence of 1 mM ATP or dATP or ATPγS.

[b] Combined DNA synthesis activity from fractions 14–16 from each HR 10/30 Superose 6 column is given. Activity assays for the eluting initiation complex were carried out by adding 3 μl of a mixture containing dATP, dGTP, dCTP, and [^{3}H]dTTP (100 cpm/pmol) to 22 μl from each fraction, incubating at 60 °C for 5 min and determining the amount of acid-precipitable radioactivity as described under "Experimental Procedures."

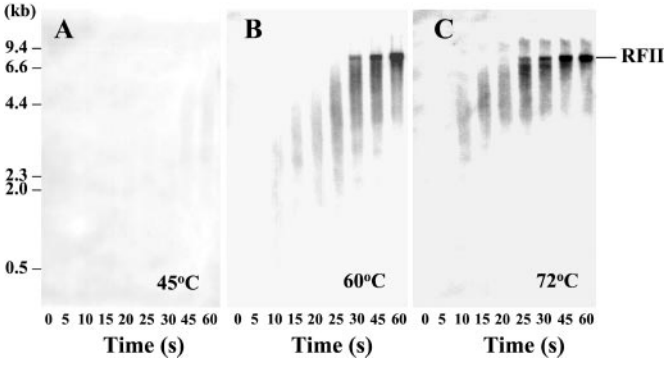

FIG. 6. **Elongation rate of the minimal *Tth* holoenzyme.** The rate of polymerization was measured by determining the time required for polymerization of the full-length M13Gori template (8623 nucleotides). Reactions were initiated by adding deoxyribonucleoside triphosphates to pre-formed initiation complexes on RNA-primed M13Gori DNA and incubating for the indicated times and then stopping the reaction by adding EDTA. DNA products were analyzed by alkaline-agarose gel electrophoresis. Reactions were carried out at *A*, 45 °C; *B*, 60 °C; and *C*, 72 °C. DNA standards are shown on the *left side* of the gels, and the full-length product is indicated on the *right side* of the gels.

45–60 s. At 60 and 72 °C, RFII is formed within 30 and 25 s, respectively. Thus, the minimal *Tth* DNA holoenzyme exhibits elongation rates of <140 nucleotides/s at 45 °C, 290 nucleotides/s at 60 °C, and 350 nucleotides/s at 72 °C.

Processivity—To test the processivity of the minimal *Tth* holoenzyme, we examined the ability of initiation complexes to synthesize DNA in the presence of primed template competitor added as a trap to prevent dissociated holoenzyme from reassociating with the M13Gori template. As a competitor template, we used activated calf thymus DNA in which single-stranded DNA regions were generated with limited nuclease treatment of sheared double-stranded DNA (3, 58). Analysis of the reaction products on alkaline-agarose gels shows that the minimal *Tth* holoenzyme indeed recognizes both activated calf thymus DNA and primed M13Gori as templates (Fig. 7, *lanes 1* and *2*). Given the difficulty of accurately estimating the molar concentration of functional primed template sites on heterogeneous species such as activated calf thymus DNA, we empirically determined that 5 μg was sufficient to completely inhibit DNA synthesis on the M13Gori template when added together during the initiation complex formation reaction (Fig. 7, *lane 3*). Thus, if the *Tth* holoenzyme dissociated from the M13Gori template, re-formation of initiation complexes on M13Gori would be prevented. When competitor template was added to the reaction mixture after the initiation complex had formed on the M13Gori template, synthesis of full-length M13Gori RFII products occurred, indicating a processivity of at least 8.6 kb

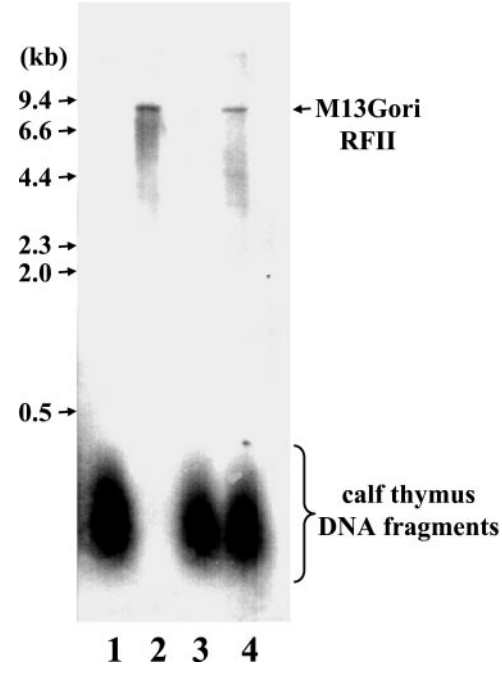

FIG. 7. **Processivity of the minimal reconstituted *Tth* holoenzyme.** To determine processivity, initiation complexes were pre-formed on RNA-primed M13Gori DNA using *Tth* holoenzyme subunits, followed by the addition of competitor DNA (activated calf thymus DNA) and deoxynucleoside triphosphates to initiate DNA synthesis reaction as described under "Experimental Procedures." The reaction was allowed to proceed for 45 s and then stopped by addition of EDTA. DNA products were analyzed by alkaline-agarose gel electrophoresis. *Lane 1*, the reaction contains only activated calf thymus DNA as a template. *Lane 2*, the reaction contains only M13Gori DNA as a template. *Lane 3*, reaction contains both M13Gori DNA and calf thymus DNA in the initiation complex formation reaction prior to the addition of dNTPs. *Lane 4*, reaction contains M13Gori as a template during the initiation complex formation step and the competitor DNA was added with the deoxyribonucleoside triphosphates.

(Fig. 7, *lane 4*). As expected, the excess subunits not associated with M13Gori were available for the synthesis of smaller (<0.5 kb) fragments resulting from their association with the competitor template (Fig. 7, *lane 4*).

DISCUSSION

In all organisms studied to date, processive and rapid synthesis of DNA by replicative polymerases requires three distinct components: polymerase core, sliding clamp processivity factor, and a clamp-loading complex. Recent identification of the α pol III catalytic subunit from a thermophilic bacterium, *Tth*, along with the associated DnaX proteins, τ and γ, suggested that the basic design of the replicative apparatus from mesophilic and thermophilic bacteria may indeed be similar. Here, we report the identification of three additional subunits of the DNA holoenzyme: δ and δ′, components of the clamp-loading complex, and the β processivity factor. To obtain sufficient protein for functional analyses, we cloned, overexpressed in *E. coli* and purified to homogeneity α, DnaX, δ, δ′, and β subunits from *Tth* as fusion proteins containing NH_2-terminal hexahistidine and biotinylation sequences. Testing of these proteins for their ability to support efficient DNA synthesis with long circular templates revealed that α, DnaX, δ, δ′, and β subunits represent the minimal essential components of the *Tth* holoenzyme. Thus, the minimal holoenzyme from this thermophilic organism contains the same essential subunits as the holoenzyme from mesophilic bacteria such as Gram-negative *E. coli* or Gram-positive *Streptococcus pyogenes* (59–61).

Similarity between thermophilic and mesophilic replicases

extends to binding interactions between the essential holoenzyme subunits. Like in *E. coli* and *S. pyogenes*, *Tth* DnaX complex proteins, δ, δ′, and τ/γ, form an assembly stable to gel filtration. The DnaX complex is held together primarily by δ-δ′ and δ′-τ/γ binding interactions. As shown previously (40) and confirmed here, *Tth* α and τ/γ subunits bind to each other as they do in *E. coli* and *S. pyogenes*. Co-migration of α, τ/γ, δ, and δ′ subunits and analysis of Coomassie Blue stain intensities of the lead fraction are consistent with the formation of a complex with apparent stoichiometry of $\alpha\tau\gamma_2\delta\delta'$. The equimolar stoichiometry of α and τ and the enrichment of τ over γ in the α-bound fractions is consistent with an exclusive α-τ interaction, just as in *E. coli* (23, 51). The DnaX-α complexes may be heterogeneous, composed of $\tau_2\gamma_1$- and $\tau_1\gamma_2$-based DnaX complexes, just like in *E. coli* (6, 7, 62, 63).

Upon incubation with a primed template and ATP, holoenzyme from *E. coli* forms an initiation complex stable to gel filtration (57). In *Tth*, an initiation complex requiring α, DnaX, δ, δ′, and β subunits and primed template is also formed in the presence of ATP (or dATP or ATPγS), but only at elevated temperature (60 °C).

Once the initiation complex is assembled, efficient DNA synthesis with the minimal *Tth* holoenzyme also requires elevated temperatures. Even at 45 °C, the DNA synthesis reaction is inefficient. At 72 °C, we have observed a maximal elongation rate of 350 nucleotides/s. This value is approaching the elongation rate of replicative polymerases from mesophilic bacteria such as *E. coli* or *S. pyogenes* of about 500–700 nucleotides/s at 30 °C (54, 59). *Tth* holoenzyme is able to polymerize the entire M13Gori template in one binding event, indicating a replicase of high processivity. It is interesting to note that even at 72 °C, the minimal holoenzyme does not produce significant levels of DNA products longer than the M13Gori template, that is, elongation stops as the polymerase completes extending the primer once around the circular template. Any local and/or partial melting of the RFII product at 72 °C (calculated T_m of M13Gori RFII is ~74 °C at the salt concentration of our assay (64)) is not sufficient to permit the polymerase to continue DNA synthesis past the original primer. Thus, rolling circle-type amplifications (65) with the *Tth* holoenzyme will probably require coupling of the *Tth* holoenzyme with a replicative helicase (21, 66).

It is likely that the efficiency of the *Tth* holoenzyme could be further enhanced by certain subunits known to be important for optimal functioning of the *E. coli* holoenzyme. Also, terminal hexahistidine/biotin tags attached to the expressed *Tth* subunits could partially interfere with their function. The minimal *Tth* holoenzyme does not contain the ε subunit proofreading 3′ → 5′ exonuclease encoded by the *dnaQ* gene in *E. coli* (59, 67). In *E. coli*, experiments with DNA holoenzyme reconstituted with τ complex, β and either α, αε of αεθ showed that the ε subunit increases the elongation rate by 2–5-fold (30, 31). In addition, the processivity of the holoenzyme reconstituted with the γ complex, but not the τ complex, is reduced in the absence of the ε subunit (31, 32, 68). Elucidation of the effect of the *Tth* DnaQ protein and other factors on the rate, processivity, and fidelity of the *Tth* holoenzyme remains an important area for future investigation.

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J. Biol. Chem. 2002, 277:13401-13408.
doi: 10.1074/jbc.M110833200 originally published online January 31, 2002
